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In vitro testing of Nd:YAG laser processed calcium phosphate coatings

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Abstract Nd:YAG laser cladding is a new method for deposition of a calcium phosphate onto metallic surfaces of interest in implantology. The aim of this study was to compare the biologic response of MG-63 human osteoblast-like cells grown on Ti-6Al-4V substrates coated with a calcium phosphate layer applied using different methods: plasma spraying as reference material and Nd:YAG laser cladding as test material. Tissue culture polystyrene was used as negative control. The Nd:YAG laser clad material showed a behaviour similar to the reference material, plasma spray, respective to cell morphology (SEM observations), cell proliferation (AlamarBlue assay) and cytotoxicity of extracts (MTT assay). Proliferation, as measured by the AlamarBlue assay, showed little difference in the metabolic activity of the cells on the materials over an 18 day culture period. There were no significant differences in the cellular growth response on the test material when compared to the ones exhibited by the reference material. In the solvent extraction test all the extracts had some detrimental effect on cellular activity at 100% concentration, although cells incubated in the test material extract showed a proliferation rate similar to that of the reference material. To better understand the scope of these results it should be taken into account that the Nd:YAG clad coating has recently been developed. The fact that its in vitro performance is comparable to that produced by plasma spray, a material commercially available for more than ten years, indicates that this new laser based method could be of commercial interest in the near future.

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1 Introduction

The biological responses to materials are influenced, both in vitro and in vivo, by the surface properties of the material used in the device [1]. In an attempt to improve the *in vivo* performance of metal surfaces, calcium phosphate (CaP) ceramics, specially hydroxyapatite (HA), have been used in a variety of clinical applications as coatings on metal prostheses, and as dense porous bone fillers for orthopaedics, neurosurgery and dentistry [2]. The exact mechanisms by which calcium phosphate ceramics elicit the reaction of the bone are not clearly understood, although it is known that the bioactivity of ceramics is related to the dissolution rate and that the early cellular response is of primary importance [3]. Cell and tissue reactions at ceramic interfaces have been extensivelly studied using cell cultures [4, 5], organ cultures [6] and animal models [7, 8]. Plasma spraying techniques are currently used for the coating of metallic prosthesis, and involve heating a ceramic powder above its melting point to produce partial molten droplets, which are accelerated by a gas stream and projected onto the metal. On impact, the droplets flow into thin lamellar particles adhering to the surface, overlapping and interlocking as they solidify. Due to

their high deposition rate, and the ability to coat large areas these techniques were commercialized very soon, but there are still major drawbacks such as low interfacial strength, low adherence of the coating (grit blasting or metal powder sintering is necessary to roughen the substrate surface before coating it), and poor control of the physico-chemical properties, and, hence, of the biological stability of the coatings [9–11].

Therefore, several studies have been carried out to optimize the technique of plasma spraying HA onto Ti6Al4V alloy [9, 12–14]. Also other techniques have been proposed to apply HA as a coating on metallic substrates such as magnetron sputtering [15], ion-beam deposition [16], electrophoretic deposition [17], pulsed laser deposition [18–21] or the so-called biomimetic methods [22–23].

The present work deals with the application of an alternative coating method, known in the metallurgical field as laser surface coating, to obtain a calcium phosphate layer onto Ti6Al4V alloy. The aim of the cladding process is to overlay one material with another to form a sound interfacial bond or weld without diluting the clad coating of which the properties are preserved [24]. The work carried out in the metallurgical field demonstrated that the coatings obtained by this technique show near isotropic mechanical properties, good fusion bonding to the substrate, and minimum surface preparation requirements. The capabilities of the laser surface cladding to produce a calcium phosphate layer onto Ti6Al4V alloy and the biocompatibility of this coating were already demonstrated in previous work [25–26].

The aim of the present study was to compare the calcium phosphate coatings produced by this new technique in the biomaterials field, laser surface cladding, and those commercially available obtained by plasma spraying. The comparative study is presented from the point of view of the biological properties.

2 Materials and methods

2.1 Substrates

Ti alloy (Ti6Al4V) plates with a thickness of 6 mm and dimensions of 50×50 mm^2 were used as substrates. The composition of the Ti6Al4V plates is as follows: 0.01% C, 0.006% H, 0.18% Fe, 6.07% Al, 3.94% V and 89.79% Ti. In order to perform the biological studies, the titanium alloy CaP coated plates (either by PS or by Nd:YAG laser cladding) were cut to smaller pieces of 10×10 mm^2 in area using an Accutom-5 cutt-off machine (Struers) with distilled water as lubricant.

2.2 Plasma spray coatings

High quality commercially PS-coatings were produced on the titanium alloy plates described above by Plasma Biotal Ltd, UK. The complete procedure included degreasing and grit blasting with alumina powder (14μm) before applying the coating.

The HA powder used to produce the PS coatings was of the same batch (Captal 60) as the one used to obtain the coatings by the laser cladding method.

2.3 Laser surface cladding coatings

Among the different techniques available to produce coatings by means of a laser, the laser surface cladding route has been selected to produce the CaP coatings. A schematic drawing of this method is shown inFig. 1. The method consists basically of blowing particles of the precursor material by mixing it with a carrier gas. This powder stream is injected into the area irradiated by the laser beam. The laser beam heats up the precursor material cloud and creates a molten pool on the metallic substrate where the particles impinge. To avoid oxidation in the interaction zone, a shielding inert gas is applied. A rapid quenching takes place when the molten pool leaves the laser irradiated area. The relative movement between the laser and the substrate leads to the formation of a coating on the surface of the titanium alloy plate.

A pulsed Nd:YAG laser (Rofin-Sinar RSY 500P) with a maximum average power of 500 W and a maximum peak power of 10 kW was used in this work. This type of laser emits infrared radiation (wavelength:1064 nm) in a way in which the pulse duration, the frequency of the pulses, and the mean power are controllable. The laser beam was coupled to an optical fibre of 400 μm core diameter and 10 m long in order to guide the energy from the laser resonator to the workpiece. The laser power at the position of the substrate was measured by means of a laser power meter (Ophir L1500-LP) coupled to an Ophir Nova reader unit. The power density obtained over the titanium alloy substrate remained at a constant value of 2000 W/cm^2, at a frequency of 40 Hz, and 4 ms of pulse duration. The transverse speed of the Ti6Al4V substrate with regard to the laser beam was set to 1mm/s.

2.4 Structural and morphological examination

Coated samples were characterized using several analytical techniques, including scanning electron microscopy (SEM), energy dispersive x-ray spectroscopy (EDX), and x-ray diffraction (XRD).

XRD analyses were carried out with a Siemens D-5000 diffractometer operating in θ–2θ geometry, using the Cu Kα radiation and the X-ray generator operating at 40 kV and 40 mA. A secondary monochromator was used on the diffracted

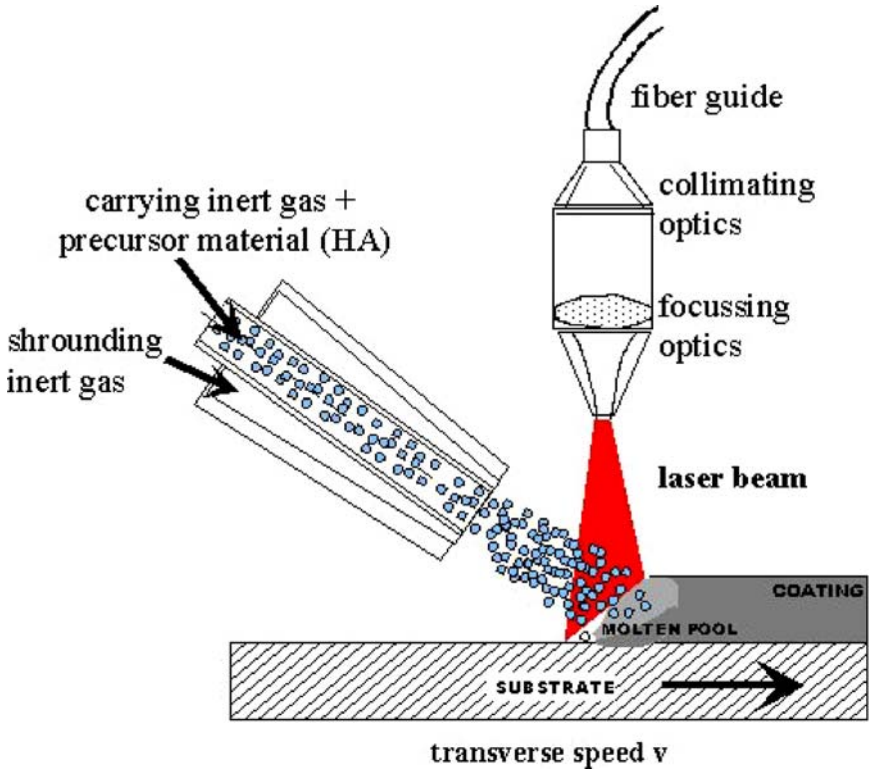

Fig. 1 Experimental setup of the laser cladding technique by blowing the precursor material

beam side with a scintillation counter detector. To determine the phase composition of the surface of the coatings, thereby reducing the influence of the substrate on the phases observed, the diffractometer was operated in grazing incidence geometry. Here, the sample (θ-circle) was fixed at a low angle (0.1–3°) almost parallel to the incident beam, whilst the detector (Siemens thin film detector) moved in a conventional 2θ circle between 20 and 50°. In this study, incidence angles of 1 and 2° were used.

Data were collected over the 2θ range 20–50° with a step size of 0.02° and a count time of 2.5 seconds. Identification of phases was achieved by comparing the diffraction patterns with International Centre for Diffraction Data (ICDD) [Joint Committe of Powder Diffraction Standard (JCPDS)] standards.

The samples were gold coated to be examined using a Philips XL30 scanning electron microscope with an accelerating voltage of 12 kV. The composition analysis was performed by means of an Edax EDX spectroscope.

To perform cross-sectional observations, the samples were embedded in Acryfix acrylic resin. They were then polished with a series of water-lubricated SiC papers up to grade 1000, followed by diamond paste finish up to 1 μm size. After this preparation, the samples were gold coated and examined by SEM, as in the case of the surface observation.

To find out which compounds and alloys might be involved in the structure of the coatings, the high-temperature phase diagrams of binary and ternary subsystems were used [27].

The surface finish of the plasma spray and laser coated samples was measured by means of a surface profilometer (Dektak^3 ST). Roughness average (Ra) values were measured from the center line of the coated plates. Measurements were taken over a 60 mm traverse of the stylus with a cut-off value of 20 mm, thus three readings were taken over the traverse ensuring that the stylus travelled over a reasonable length.

2.5 Cell culture conditions

The MG-63 human osteoblast-like cell line (ATCC number CRL 1427) was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were regularly cultured in Earle's modification of Eagle's Medium (EMEM) supplemented with 10% of foetal calf serum, 1% antibiotics, L-glutamine and vitamin C. Tissue culture polystyrene (TCP) was used as a negative control. Calcium phosphate coated test samples (either by PS or by Nd:YAG laser cladding) of 10 mm × 10 mm in area and 6 mm thick were placed, under sterile conditions, in 24-well TCP plates. The sterilisation procedure of the pieces was performed by dipping the specimens in a 70% ethanol solution and further air drying. This procedure allowed cell growth without detectable contamination signs in 18 days experiments. To assess the proliferation capacity of the cells on the different substrates, which would allow further comparisons among them, a micro-mass culture technique was used to obtain cell growth only on the surface area of the testing

substrates and not on the surrounding polystyrene surface. Each piece was seeded with 50,000 cells in a 50 μl drop. Cells were allowed to adhere for 2 hours, covered with 1 ml culture medium, and incubated at 37°C, in 5% CO_2, for different periods of time. For cells growing directly on TCP the same procedure was followed. After each incubation period, the test samples were processed for SEM observations or submitted to a cell proliferation test.

2.6 Scanning electron microscopy observation of cell cultures

Three replicas calcium phosphate coated test samples (either by PS or by Nd:YAG laser cladding) were used to grow MG-63 osteoblast-like cells and were compared at 3, 6 and 24 hours and 6 and 18 days using SEM. After each incubation period the test samples were rinsed three times with phosphate-buffered saline (PBS) and fixed with 2,5% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours. After fixation, the test samples were washed three times with PBS and sequentially dipped in 30, 50, 70, 80 and 95% ethanol for 30 min each and in 100% ethanol for 1 hour. The pieces were submitted to critical point-drying in an increasing ethanol-amylacetate mixture (3:1, 1:1, 1:3, 15 min each) and in pure amylacetate for 15 min, twice, and finally vacuum-dried. A thin layer of gold was sputter-coated onto the pieces prior to examination in a Philips XL30 scanning electron microscope.

2.7 Cell proliferation test

The number of cells growing on the coated substrates (by PS or by Nd:YAG laser cladding) and on the negative control TCP (Tissue Culture Polystyrene) was quantified by assessing their cellular activity at 1, 6, 12 and 18 days of culture by performing the alamar Blue™proliferation assay (Serotec Ltd). Alamar Blue™ is a REDOX indicator that changes colour in response to the chemical reduction of the culture medium which results from cell growth and division. The alamar Blue™ can then be removed and replaced with fresh medium so that monitoring can be continued. A sample size of six was used in each experimental group. After each incubation period, cultures were removed from incubator, an amount of alamar Blue™ reagent equal to 10% of the culture volume was aseptically added, and cultures were returned to the incubator for 4 hours. Each well content was saved and replaced with fresh culture medium. A 100 μl aliquot of every sample was placed in a 96-well microtiter plate and the absorbance of the samples was measured using a Bio-Rad Model 550 microplate reader. The wavelength to measure the absorbance of the dye product was 570 nm while the subtract background absorbance was 630 nm.

2.8 Solvent extraction test

One test sample of each of the coated substrates was extracted by rolling at 37°C for 90 hours in EMEM culture medium supplemented with 10% foetal calf serum. A surface area to volume ratio of 0.33 cm^2/ml was used. To use as a control, TCP was extracted in the same conditions. After the incubation, every extract was sterilised through a 0.22 μm pore size filter and its pH value was measured with a CRISON micro-pH 2001 pH-meter.

MG-63 osteoblast-like cells were grown to confluent layers in 96-well tissue culture plates in a final volume of 1 ml of culture medium per well. Different concentrations (5, 10, 20, 50 and 100%) of the extracts were incubated with cells for 24 hours. Three wells per substrate and extract concentration were used. The cellular activity was quantified using the Cell Proliferation Kit I (MTT) from Roche Molecular Biochemicals. After the incubation period, 10 μl of the MTT labelling reagent (3-[4,5-dimethyltyazol-2-yl]-2,5-diphenyl tetrazolium bromide, in PBS) was added to each well at a final concentration of 0.5 mg/ml. Cells were incubated for 4 hours under culture conditions (37°C, 5% CO_2, humidified atmosphere) to allow the production of purple formazan crystals. In order to dissolve these, 100 μl of a solution containing 10% SDS in 0.01 M HCl, was added to each well allowing the 96-well plate to stand overnight under culture conditions. The absorbance of the samples was measured using a Bio-Rad Model 550 microplate reader. The wavelength to measure absorbance of the formazan product was 570 nm and the reference wavelength was 700 nm.

2.9 Quantitative data analysis

Mean values obtained from the cell activity on CaP coated samples (either by PS and Nd:YAG laser cladding), and from TCP (negative control) in cell proliferation and solvent extraction tests were compared and assessed for significant differences using the computer program SPSS for Windows (v 9.0). Statistical analysis consisting of a one-way analysis of variance and pair-wise multiple comparison tests (Student Newman-Keuls) were used to determine if significant differences existed between the mean values of the experimental groups. Differences between groups were considered significant at $p < 0.05$.

3 Results and discussion

In order to better understand the response of the osteoblast-like cells to the CaP coatings produced by Nd:YAG laser cladding, it is important to take into account two factors: the topography of the coating surface and the chemical

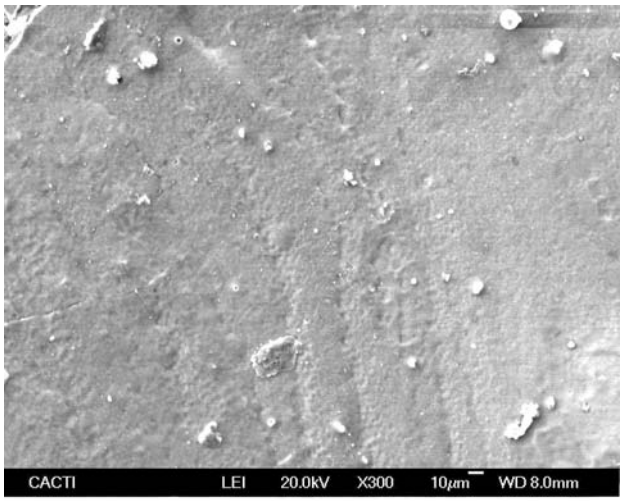

Fig. 2 SEM micrographs showing the surface topography of the coatings produced by the Nd:YAG laser cladding technique (a) and that of the coatings obtained by plasma spraying (b)

composition of those coatings.Figure 2 shows the typical aspect of the surface of a CaP coating obtained by Nd:YAG laser cladding (Fig. 2a) and that of the CaP coatings produced by plasma spraying (Fig. 2b). As can be clearly seen, the coatings obtained by Nd:YAG laser cladding are smoother than those produced by PS. Quantitative roughness measurements of those surfaces gave Ra values of 7,7 μm for the coatings produced by the laser cladding technique while those obtained by PS gave a higher Ra value of 22,0 μm.

Regarding the chemical composition of the coatings, the different analytical techniques used to characterize them revealed that the coatings produced by Nd:YAG laser have a composition that varies across its thickness [28]. Starting from HA and HA glass at the surface to a mixture of α-TCP, HA and HA glass inside the coating and ending with calcium titanates and titanium phosphides at the coating-substrate interface. This composition allows assuring the two main objectives of the coating: the inner part of the coating acts as a bonding agent to the implant, while the outer part acts as a bioactive material.

To evaluate the cell attachment and proliferation, three replicas from CaP coatings produced either by Nd:YAG laser cladding and by PS were compared at 3 and 6 hours, and 6 and 18 days by SEM. Proliferation was also measured by the alamar Blue^{TM} assay. MG-63 osteoblastic cells seeded on the surface of the PS and Nd:YAG coatings proliferated very well and presented a normal morphology(Figs. 3 and 4). At early stages cells have adhered to the surface of both materials (Fig. 3), showing either a flat morphology, making their nuclei visible, or a rounded morphology due to the events involved in cellular division. After 6 days of growing, cell layers were observed on both materials and after 18 days cell layers were thick. The CaP coatings obtained either by Nd:YAG laser cladding or by PS were susceptible to being colonised by the osteoblastic cells at a similar rate. The values observed for the alamar Blue^{TM} reduction (Fig. 4) were higher in control cultures suggesting that an increased number of cells attached to the TCP plates as compared with that observed on the material surfaces. There were no statistically significant differences in the proliferation of MG-63 cells on the Nd:YAG coating when compared to the values exhibited by PS over a 18 days culture period.

The solvent extraction test shows a pH change. The pH value of the EMEM at 37°C prior to extract preparation was 7.49.Table 1 contains the results of measuring the pH values of 100% extract concentrations after 90 hours of rolling at 37°C. The increase of the pH value of the pure EMEM solution during the 90 hours extract preparation should be noted. The pH value of the CaP coating produced by Nd:YAG laser pieces is more basic than the control one, while the values obtained for the reference material (CaP coating obtained by plasma spraying) is slightly lower than that of the EMEM.

The MTT assay results are presented relative to the cellular activity of the negative control (TCP), i.e. the horizontal line at value 1 on the y-axis, (Fig. 5) and show that both materials had only some effect at 50 and 100% of eluate concentration, none of them being statistically significant.

As shown by Fig. 2 and corroborated by the surface roughness measurements, the coatings obtained by Nd:YAG laser cladding are smoother than those produced by PS. This factor must be taken into account when comparing the biologic results because a coating having a larger surface will be able to locate a larger number of cells, therefore the slightly higher value of cell proliferation obtained by the PS CaP coatings could be related to this factor.

On the whole, the results of the experimental work performed, allow to affirm that the CaP coatings produced by Nd:YAG laser cladding showed a behaviour similar to

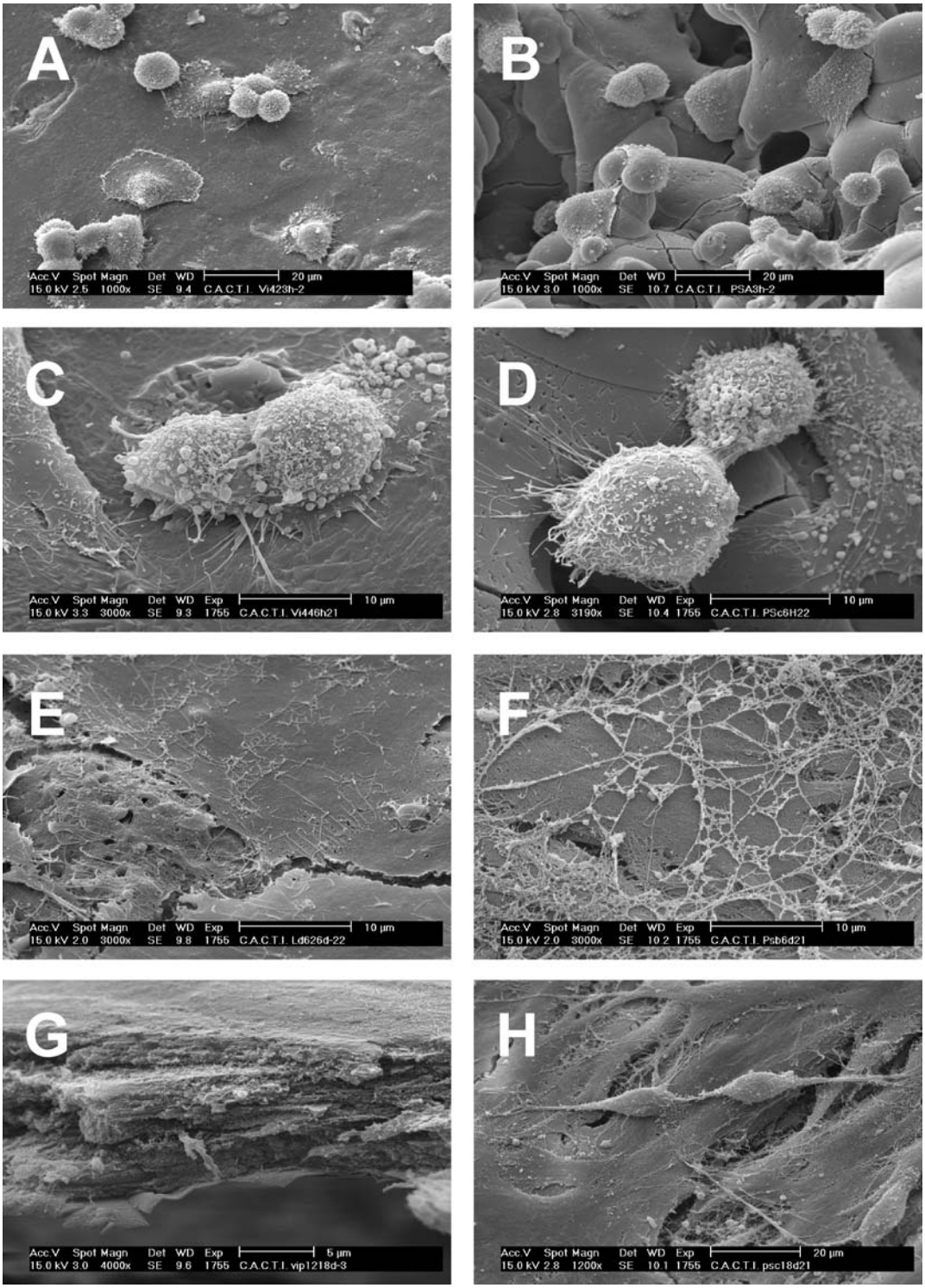

Fig. 3 SEM micrographs showing the cell morphology at 3 hours (A and B), 6 hours (C and D), 6 days (E and F) and at 18 days (G and H) after seeding of CaP coatings produced by Nd:YAG laser (A, C, E and G) and CaP coatings produced by plasma spray (B, D, F and H)

the reference material (CaP coatings produced by Plasma Spray) respective to cell attachment (SEM), cell proliferation (alamar Blue^{TM} assay) and cytotoxicity of extracts (MTT assay).

4 Conclusions

Calcium phosphate coatings obtained by a novel technique, the Nd:YAG laser cladding, have been compared from the

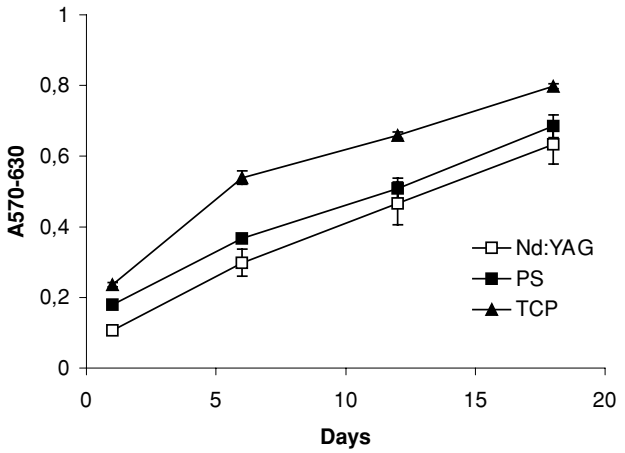

Fig. 4 Proliferation of the MG-63 osteoblast-like cells in terms of absorbance values obtained by means of the Alamar Blue cell activity assay (Nd:YAG: CaP coatings produced by Nd:YAG laser; PS: CaP coatings obtained by plasma spray; TCP: tissue culture polystyrene used as control material)

Table 1 pH values of 100% concentrated eluates

Sample	pH value
EMEM (negative control)	8.53
CaP coating applied on Ti6Al4V by Nd:YAG laser cladding	8.84
Ti6Al4V without coating	8.37
CaP coating applied on Ti6Al4V by plasma spray	8.41

point of view of the biologic response of osteoblast-like cells, to commercial calcium phosphate coatings produced by the plasma spraying technique but using the same precursor material.

The calcium phosphate coatings obtained by the Nd:YAG laser cladding technique showed a behaviour similar to the reference materials, Ti-6Al-4V alloy and CaP coatings produced by plasma spray, respective to cell morphology (SEM observations), cell proliferation (AlamarBlue assay) and cytotoxicity of extracts (MTT assay). To better understand the scope of these results it should be taken into account that the Nd:YAG clad coating has recently been developed. The fact that its in vitro performance is comparable to that produced by plasma spray, a material commercially available for more than ten years, indicates that this laser based method is very promising.

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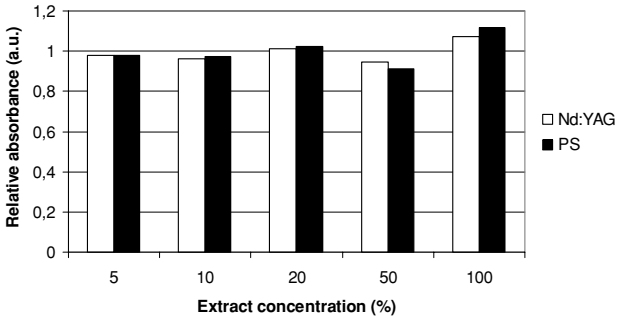

Fig. 5 MG-63 osteoblast-like cellular activity after incubation with different concentrations of extracts prepared from de CaP coatings produced by Nd:YAG laser cladding (Nd:YAG) or by plasma spray (PS)

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